

Inter-laboratory Testing of GMO Detection by Combinatory SYBR®Green PCR Screening (CoSYPS)

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Received: 13 December 2013 / Accepted: 3 March 2014 / Published online: 26 March 2014
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Abstract Combinatory SYBR®Green real-time PCR Screening (CoSYPS) is an efficient, sensitive approach for detecting complex targets such as genetically modified organisms (GMOs) in food and feed products. GMO analysis for legal purposes has become increasingly complex and costly due to the diversity in recombinant targets present in the different GMOs. For this reason, screening for the presence of GMOs is in general the first step in the detection of GM material in a product. CoSYPS allows detecting the large majority of globally commercial GMOs using SYBR®Green real-time PCR methods for six GM targets (P35S, Tnos, CryIAb, CP4-EPSPS, PAT and BAR) combined with species-specific PCR methods (e.g., maize, soy, rapeseed). Here, the results of an inter-laboratory trial on seven samples with different GMO mixtures at different levels are presented. In total, 13 laboratories participated in the trial and the currently most frequently used PCR analysis platforms are represented. The inter-laboratory study clearly demonstrates that PCR methods used in CoSYPS form a very robust GMO screening system. Sensitivity, specificity, positive and negative predictive values are for all PCR methods higher than 95 % for all samples. Together, these results show that the SYBR®Green real-time PCR methods used in CoSYPS are effectively applicable to different PCR platforms and amendable to configuration into a sensitive high-throughput GMO screening and decision support tool.

Keywords Combinatory SYBR®Green PCR Screening · GMO detection · Genetically modified food

Introduction

Genetically modified (GM) food and feed products have become a reality today (James 2012). Since their initial commercialization in 1994, about 150 genetically modified plants have received approval for use as food or feed (<http://cera-gmc.org>). In most countries, such use is strictly regulated and compliance measures have been implemented. An essential element in the enforcement of this legislation is the efficient detection of genetically modified organisms (GMOs) or traces thereof in food and feed products (<http://gmo-crl.jrc.ec.europa.eu/gmomethods>).

Considering their large diversity (different GM elements in various species), the application of a generic screening for the presence of GM material is most often the first step in GMO analysis (Holst-Jensen et al. 2012). Such qualitative screening methods provide a presence/absence response (taxon-specific sequence or a GM element) and help reducing the number of subsequent identification analyses.

For this reason, screening for the presence of GMOs is in general the first step in the detection of GM material.

In most cases, GMO screening approaches apply quantitative PCR (qPCR) methods for detecting the presence of GM material in food and feed samples. More complex strategies employing microarrays (e.g., GMOchips (Leimanis et al. 2006), NAIMA (Morisset et al. 2008), and PADLOCK probes ligation (Ujhelyi et al. 2012)) have been attempted, but are, however, still under validation (NAIMA, padlock approach) or received limited distribution within the enforcement world (GMOchips). Thus, today, qPCR screening is by far the most commonly applied approach in screening for GM materials in food and feed products. A set of inter-laboratory trial validated

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GMO detection methods have been compiled into a Compendium (Van den Eede 2010). The most common qualitative qPCR methods used for screening are probe-based qPCR methods (Fernandez et al. 2005; Lee et al. 2007; Waiblinger et al. 2010). The PCR methods described in the Compendium were designed by many different groups and, as a consequence often, do not apply the same PCR conditions and protocols. As such, a combined use of these methods in a single run in a 96 well plate format may not be feasible without prior verification of the performance and efficacy in such usage (Morisset et al. 2008).

To meet the need of a sensitive, reliable but also cost-effective and flexible GMO screening strategy, a set of simplex SYBR®Green qPCR GMO detection methods which all can be run together in a single analytical PCR plate has been developed at the Scientific Institute of Public Health (Barbau-Piednoir et al. 2010; Barbau-Piednoir et al. 2012; Van den Bulcke et al. 2010; Mbongolo Mbella et al. 2011). Six GM-element SYBR®Green qPCR screening methods were newly developed for this purpose: four trait-specific methods (CP4-EPSPS, CryIAb, PAT, BAR), and two methods detecting the major GM generic recombinant elements, the cauliflower mosaic virus (CaMV) 35S promoter (p35S) and the *Agrobacterium* Nos terminator (tNos) (Barbau-Piednoir et al. 2010; Barbau-Piednoir et al. 2012). These GM-element-specific methods were to be combined with SYBR®Green taxon/species-specific qPCR methods targeting e.g.; a lectin, an alcohol dehydrogenase, and a cruciferin target DNA sequence for determining the presence of material derived from soybean, maize, and rapeseed, respectively (Mbongolo Mbella et al. 2011). In addition, a method detecting a generic plant marker, the chloroplastic RbCl element (Mbongolo Mbella et al. 2011) and a CaMV-specific method to demonstrate the possible presence of CaMV material in a sample, were included. The combined use of both sets of qPCR methods and a decision support system (DSS) allows performing a GMO screening analysis within one run on a single PCR plate and was designated “Combinatory SYBR®Green qPCR Screening” (CoSYPS) (Van den Bulcke et al. 2010). In the DSS, the outcome of analyses using the SYBR®Green qPCR methods is interpreted using a DSS that applies a “prime number”-based algorithm to indicate which GMOs are possibly present in the sample (Van den Bulcke et al. 2010).

It was shown in-house that all these SYBR®Green qPCR GMO screening methods performed equally well under the same reaction conditions with high specificity and sensitivity on different qPCR platforms (own unpublished results). To complete the validation of CoSYPS in GMO analysis, an inter-laboratory trial was organized to evaluate the performance in different laboratories and evaluate the general applicability of SYBRGreen technology considering the lesser experience of most laboratories with this type of qPCR.

Multiple test samples were prepared including various GMOs (soy GTS40-3-2, maize MON 810 and Bt11, and rapeseed RF3 and RT73) at different levels (0–2 %) in different backgrounds (soy, maize and rapeseed). Participants were provided with the test samples, the test protocol, all reagents and the CoSYPS DSS. Here, the results of the CoSYPS inter-laboratory trial are presented. The significance, practical bottlenecks and future opportunities of the use of CoSYPS in GMO analysis are discussed.

Materials and Methods

Materials

Plant gDNA was extracted from leaf tissue of in-house grown plants (WT maize, soy and rapeseed; Bt11 and MON 810 GM Maize; GTS40-3-2 soy) or from CRM powders of the Institute for Reference Materials and Measurements (IRMM) (MON 810 GM maize) or as purified DNA of the American Oil Chemists’ Society (AOCS) (GT73 and MS8/RF3 rapeseed).

For the in-house plant tissue production, standard growth conditions were at 16/8-h day/night regime at 25 °C with chamber humidity at 80 % in a Schneider Scientific Plant growth chamber (S1084).

All Sybricon plasmids used as positive controls were constructed in-house, isolated using Qiagen mini/midi plasmid preparations and verified by DNA sequence analysis (Barbau-Piednoir et al. 2010; Barbau-Piednoir et al. 2012; Mbongolo Mbella et al. 2011). Plasmid DNA concentration was measured by Spectrophotometry (Smart Spec Plus; Bio-Rad), diluted in two steps at 5 ng/μl in DNase/RNase free water (Acros). This dilution was requantified with Picogreen (Invitrogen). Then a second dilution at 5 fg/μl was prepared, in two steps, from the dilution at 5 ng/μl by dilution in carrier DNA (Calf Thymus DNA 4 ng/μl). Plasmid dilutions were verified by qPCR using the appropriate SYBR®Green qPCR methods corresponding to each of the targets (data not shown).

As negative control, DNase/RNase free water (Acros) was used and indicated as “No Template Control” (NTC).

General Methods

Genomic DNA Isolation and Quantification

Total DNA extraction of the different materials (seeds, powders, leaves) was performed as described in (Barbau-Piednoir et al. 2010). In short, 250 mg to 1 g homogenized material was extracted by a CTAB DNA extraction protocol. The final genomic DNA (gDNA) pellet was resuspended in 200 μl DNase/RNase-free water (ACROS) and stored at –20 °C.

The extracted gDNA was quantified using the PicoGreen dsDNA quantitation kit (Invitrogen) on a VersaFluor™ Fluorimeter (Bio-Rad).

Primers, Probes and PCR Reagents

All primers applied in the SYBR®Green qPCR methods were as described (Barbau-Piednoir et al. 2010, 2012; Mbongolo Mbella et al. 2011) (see Table 1). Endogene Taqman® qPCR methods applied in the EURL-GMFF validated GMO detection methods were used to identify possible traces of contamination by other crops: for maize, the ADH reference gene PCR detection method from the Bt11 quantitative method (<http://gmo-crl.jrc.ec.europa.eu/summaries/Bt11-protocol.pdf>); for soybean, the lectin reference gene PCR detection method from the GTS40-3-2 quantitative method (http://gmo-crl.jrc.ec.europa.eu/summaries/40-3-2_validated_Method.pdf), for oilseed rape, the MDB reference gene PCR detection method from the Gt73 quantitative method (http://gmo-crl.jrc.ec.europa.eu/summaries/RT73_validated_Method.pdf), for rice, the KVM reference gene PCR detection method from the LL62 quantitative method (http://gmo-crl.jrc.ec.europa.eu/summaries/LLRICE62_validated_Protocol.pdf), and for cotton, the acp1 reference gene PCR detection method from MON 531 quantitative method

(http://gmo-crl.jrc.ec.europa.eu/summaries/MON531_validated_method.pdf).

All primers and probes were purchased at Eurogentec. Mastermixes were purchased at Diagenode.

In-house Quality Verification and Unknown Preparation

The quality of the DNA extraction was estimated on the basis of two criteria: 1) the extracted DNA quantity and 2) the purity of the gDNA extracted (no contamination by unexpected targets). Extracted DNA quantity needed to be concentrated enough to be able to prepare the samples for the 13 laboratories.

qPCRs were carried out on ABI 7300 PCR System (Applied Biosystems) using the SYBR®Green qPCR Mastermix (Diagenode).

Taqman® qPCR contamination checks were performed on an ABI 7300 PCR System (Applied Biosystems) using the TaqMan® PCR Mastermix (Diagenode).

The purity of the gDNA extract was checked as described above. Trace amounts of cross-contaminating GM material could not be avoided 100 % in all preparations though. Neither the nature nor the source of the contaminating GM material was determined in further detail but late positive

Table 1 Description of the SYBR®Green qPCR methods applied in the GMO screening

PCR method	Name	Amplified gene target	Amplicon length (bp)	Target class	Reference
Plant	VPRBCP1 VPRBCP1	Ribulose-1,5-bisphosphate carboxylase/oxygenase	95	Plant kingdom	Mbongolo Mbella et al. 2011
STLM (lectin)	SLTM 1 SLTM 2	Lectine	81	Soy specific	Mbongolo Mbella et al. 2011
ADH alt	ADH-alt Fwd ADH-alt Rev	Alcohol dehydrogenase 1 promotor	83	Maize specific	Mbongolo Mbella et al. 2011
CRU	Cru770 F Cru770 R	Cruciferine	85	OSR specific	Mbongolo Mbella et al. 2011
P35S short	35S_N3Fwd 35S_N3Rev	35S RNA promoter (CaMV)	75	Generic recombinant	Barbau-Piednoir et al. 2010
Tnos	tNOS_NN_Fwd tNOS D Rev	Terminator nopaline synthase (<i>Agrobacterium</i>)	69	Generic recombinant	Barbau-Piednoir et al. 2010
CP4 epsps	CP4 Synthetic F CP4 Synthetic R CP4 synth Rbis	EPSPS CP4 EPSPS RRS Gt73 EPSPS	108	Trait specific	Barbau-Piednoir et al. 2012
CryIAb	CryIAb_Bt.Cott_Fwd CryIAb_Bt.Cott_Rev	Endotoxine delta (<i>Bacillus thuringiensis</i>)	73	Trait specific	Barbau-Piednoir et al. 2012
PAT	Pat-Pat Fwd Pat-Pat Rev	Phosphinothricin- <i>N</i> -acetyl transferase (<i>Streptomyces viridochromogenes</i>)	109	Trait specific	Barbau-Piednoir et al. 2012
BAR	Pat-Bar Fwd Pat-Bar Rev	Phosphinothricin- <i>N</i> -acetyl transferase (<i>Streptomyces hygrosopicus</i>)	69	Trait specific	Barbau-Piednoir et al. 2012
CRT 2	CRT-F CRT-R2	Reverse transcriptase of CaMV	94	Donor Organism marker	Adapted from Wolf et al. 2000

signals ($C_t > 35$) were obtained for some GM markers (p35S, tNos, CP4-EPSPS, CryIAb and PAT) and for the maize and soybean plant species markers (data not shown). For this trial, a gDNA extract was considered acceptable when the C_t observed from the impurities was above 35 C_t . All acceptable gDNA fractions were pooled, mixed overnight on a rotating wheel, diluted at 2 ng/ μ l and used for preparing the respective 7 ml of unknown in v/v ratios.

Statistical Data Analysis

Sensitivity, specificity, false positive and negative rates were calculated for all methods and samples along with the positive and negative predictive values (<http://www.cebm.net/index.aspx?o=1042>). Confidence intervals (P (95 %), $\alpha=0.05$) were calculated according to Newcombe (1998). Pearson chi-square analysis ($\alpha=0.05$) was performed using STATA Statistics/Data Analysis version 10.1.

Inter-laboratory Trial Methodology

General Organization

A large-scale (LS) collaborative trial was organized jointly by WIV-ISP and the EURL-GMFF. The study was launched on 17 June 2009 and all experimental work by the participating laboratories was to be finalized within 4 weeks after receipt of the materials. During this period, the laboratories could contact the WIV-ISP GMOLab for technical assistance. An official invitation was sent to all laboratories of the European Network of GMO Laboratories (ENGL) ($n=96$) by the EURL GMFF. Twenty-three laboratories, belonging to 17 countries of the EU, responded positively and from this group 13 laboratories were selected at random. All participating laboratories were requested to sign a Material Transfer Agreement prior to sending off of the test materials. All selected laboratories received ten unknowns (U1 to U10), the CoSYPS inter-laboratory protocol, the Excel interpretation matrix files (DSS), and the Deviation Report Form. After the experimental work, all participating laboratories were requested to complete an information sheet to document the technical infrastructure of the laboratory and their experience with fulfilling the CoSYPS system protocol.

Aim and Design of the Large-Scale CoSYPS Inter-laboratory Trial

The aim of this study was dual: first, to demonstrate a “proof of concept” of CoSYPS to detect various levels of GMOs in different backgrounds and the evaluation of pre-fixed “cut-off values” and the CoSYPS DSS; and second, to demonstrate the general applicability of CoSYPS to estimate the impact of introduction of SYBR®Green qPCR analysis and the potential

adaptive actions to take in the context of implementation of the CoSYPS methodology in a new laboratory.

For this, three control (maize, soy or rapeseed material) and seven GM test samples, the latter further indicated as GM unknowns, were prepared in-house by the WIV-ISP (see Table 2). As positive control, a plasmid DNA mix was prepared with ± 40 copies/ μ l of each of the so-called “Sybricon” plasmids. Each Sybricon contains the amplicon produced by the corresponding SYBR®Green real-time qPCR method (Barbau-Piednoir et al. 2010).

The proof of concept for CoSYPS analysis was tested by comparing the outcomes of the laboratory tests with the expected true values. Evaluation of the inter-laboratory data was performed per GM unknown ($n=7$), per qPCR method ($n=11$) and combined for all seven unknowns.

Preparation of Sample Panel and Quality Assessment

For the preparation of the GM unknowns, a set of GMOs was chosen to cover every GM marker in CoSYPS at least once (GM markers present in each particular GMO are indicated between brackets; see also Table 2): GM maize Bt11 (P35S, Tnos, CryIAb, PAT) and MON810 (P35S and CryIAb), GM soy GTS40/3/2 (P35S, Tnos, CP4 epsps), and GM rapeseed GT73 (CP4 epsps) and RF3 (Tnos, BAR).

An aliquot of each unknown sample was tested by the CoSYPS qPCR method for further characterization and verification at the WIV-ISP, prior to send off (data not shown). These outcomes served as a reference in the statistical/heuristic assessments and were used to assess the Excel DSS file.

During this quality assessment performed by the WIV-ISP, the presence of GMO traces in some wild type maize and soybean materials was observed, but at C_t levels above the usual in house cut-off value for routine testing (cut off set at $C_t=35$). This cut-off represents a rounded average C_t value (minus 1 C_t to cover a 2-fold PCR efficiency decrease due to, e.g., matrix inhibition effects). This average C_t value was calculated prior to the inter-laboratory trial from the C_t values corresponding to the lowest number of copies detectable by each of the GM methods applied in screening analysis, wherein “lowest number of copies detectable” was defined as obtaining one out of two positives when two repeats are applied in a serial dilution analysis of genomic certified reference material with known zygosity for the respective targets (data not shown).

In addition, presence of CaMV was observed in all the samples containing AOCS rapeseed material (positive results with the CaMV-specific target qPCR method) whereas the samples prepared with in-house produced rapeseed material were negative (data not shown). Due to this difference with regard to the origin of the reference material, it was decided

Table 2 Composition of the test samples (U1 to U10) used in the inter-laboratory studies

	Plant materials (mass%)	Plant	Adh alt	STLM (lectine)	CRU	P35S short	T-nos	CP4- EPSPS	CryIAb	PAT	BAR	CRT 2
U1	100 % maize	+	+									
U2	100 % OSR	+			+							
U3	100 % soy	+		+								
U4	0,1 % GTS40-3-2 soy in 100 % soy	+		+		+	+	+				
U5	2 % Bt11 maize in 100 % maize	+	+			+	+		+	+		
U6	0,5 % MON 810 maize in 100 % maize	+	+			+			+			
U7	0,1 % GT73 OSR in 100 % OSR	+			+			+				
U8	0,9 % RF3 OSR in 100 % OSR	+			+		+				+	
U9	2 % RF3 OSR, 1 % GTS40-3-2 soy in 100 % maize	+	+	+	+	+	+	+			+	
U10	0,1 % GT73 OSR, 0,5 % MON 810 in 40 % OSR and 60 % maize	+	+		+	+		+	+			

The markers are as described in Table 1. *Plus symbol (+)* stands for “present in the GMO”; *minus symbol (–)* denotes “not present in the GMO”

not to include the results obtained with the CaMV-specific method.

Procedure for qPCR

All participants received a full analytical procedure together with the samples. No training in advance was given to the participants. A standard 25 μ l reaction volume was applied containing 5 μ l of template (wherein or 2 ng/ μ l gDNA or 40 copies plasmid DNA/ μ l were included), 1 \times SYBR[®]Green qPCR Mastermix, and 250 nM of each primer (except RbCl method which used final primer concentration of 1.6 μ M for VPRBCP1 and 1.2 μ M for VPRBCP2). The thermal program consisted of a single cycle of DNA polymerase activation for 10 min at 95 °C, followed by 40 amplification cycles of 15 s at 95 °C (denaturing step) and 1 min at 60 °C (annealing-extension step). After completion of the run, a melting point analysis was performed by stepwise temperature increase (± 1.75 °C/min) from 60 °C to 95 °C over 20 min.

Acceptance Criteria for Positive qPCR Test

A qPCR test was considered “positive” when the observed C_t value was lower than 35 in combination with an acceptable T_m value related to the T_m value of the positive control for the particular qPCR test (see also below). The acceptance of the T_m value from the positive controls were compared to the outcome of the in-house qPCR device testing and accepted with a maximum deviation of 1 °C. For the T_m values of the sample, the T_m value was set as positive when it differed less than 1 °C from the T_m of the positive control, except for two targets. For the CryIAb target, two different GM sequences have been engineered giving rise to two distinct melting peaks for the same target region. For this reason, the acceptable T_m range for CryIAb was set to ± 2 °C. For CP4 epsps, also two

different GM sequences have been engineered, three primers (two forward and one reverse) were designed, amplifying the respective engineered GM targets also resulting in two different melting peaks. Here, the acceptable T_m range was set as -1 °C from the lowest T_m value to $+1$ °C of the highest T_m value of the two positive controls.

Decision Support on Interpretation of the qPCR Results

Each qPCR analysis was performed in six folds on each sample. In-house CoSYPS LOD levels were determined in a six-fold repeat analysis of a target dilution series (Barbau-Piednoir et al. 2010). Here, three decision levels were distinguished: “Above LOD” (at least five out of six positives), “At LOD” (four out of six positives) and “Below LOD” (less than four out of six positives). The outcome for each method is then combined into a Gödel Prime number Product (Van den Bulcke et al. 2010) and interpreted using the prime-number GMO matrix table included in the CoSYPS DSS using a factorization algorithm (Van den Bulcke et al. 2010).

Results

General Evaluation of the CoSYPS Inter-laboratory Trial

All participants finalized the analyses within the requested 4-week period. Six laboratories requested additional information and three required supplementary material. The main questions related to the trial organisation were essentially due to a lack of familiarity with the SYBR[®]Green technology and issues with DSS when using other qPCR devices platforms than an ABI-type. Technical issues resulting in partly missing data were observed with eight of 130 tested plates.

At the end of the trial, a survey was performed to record the setup used in the different laboratories (qPCR device and software, PCR plates, sealing type) and to evaluate the general appreciation by the participants of the use of the CoSYPS. The majority of the laboratories (69 %) used an ABI-type qPCR machine. Most laboratories (92 %) experienced the CoSYPS system as easy to understand/perform and user-friendly (77 %). All laboratories were satisfied with the included protocols, although 31 % of the participants considered the transfer of the analytical results into the CoSYPS Decision Support System as “tedious”.

It can be concluded that in general the CoSYPS trial was well executed and all participants were satisfied with the protocol and the CoSYPS Decision Support System, except for the data transfer into the CoSYPS Excel DSS file.

Description of the Applied Adaptative Actions on the LS Inter-laboratory Raw Data Sets

As the results were communicated and analyzed in an Excel format, a first trouble shoot analysis was performed based on these datasheets. The data obtained with the ABI 7500–7500 software V2.0 could not be interpreted using the provided formats and an adapted version had to be developed (data not shown). For the other platforms applied in the LS trial, namely, ABI 7300, 7700 and 7900, the LC480 Roche, the Stratagene X3000 and the Bio-Rad iQ5, the provided Excel DSS were functioning appropriately.

A second adaptation was done in the context of the inappropriate setting of the threshold value. The results obtained for each unknown by each laboratory were then regrouped in tables and ranked per unknown and per method (data not shown). From this analysis, it was observed that 1) in some samples from the maize and soy wild material, traces of GMOs (but above $C_t=35$) were present (source of false positives and 2) in a number of GM unknown samples, some GM markers were detected at levels just above the cut-off value (resulting in false negatives). The scoring by some laboratories of some positive GM markers just above the cut-off value was re-analyzed as described above. In total 63 analyses (=4.4 %; 63/1430) were re-evaluated by the same person at IPH and for 22 runs (=1.85 %) an adaptation was introduced. In all these cases, the threshold was indeed not set as explained in the protocol.

Finally, one setup failed to correctly detect both melting peaks for the CP4-EPSPS positive plasmid controls. In their analysis the CP4 epsps positive control yielded only one of the two expected peaks in melting analysis (resulting in a “below LOD” conclusion for the second melting peak, if present). A re-analysis was tried to be performed but the raw data were not readable, this probably due to incompatibility with the software versions available. For this, signals with T_m values

falling within the limits of the PCR device type were accepted as positives.

Sensitivity, Specificity, and the Positive and Negative Predictive Value of the CoSYPS SYBR®Green qPCR Methods in the LS Inter-laboratory Trial

The outcomes of the CoSYPS analyses of ten markers of the seven GM unknowns are summarized in Table 3. The sensitivity or specificity for each method in each sample separately was calculated. In case a marker was expected to be positive, the sensitivity was reported, in case the marker was expected to be absent, the specificity was reported. The results show that all separate SYBR®Green qPCR methods performed appropriately in detecting their respective target(s) as in quasi all samples levels >95 % are obtained for sensitivity or specificity. The lower specificity of the soy marker (specificity at 94 % in some samples) is due to the presence of a small soy contamination in some of the samples (at the cut-off value), detected due to the high sensitivity of the method.

In Table 4, four parameters were calculated for the ten markers of the seven GM unknowns: sensitivity, specificity, positive and negative predictive value. In the case of GMO screening applications, especially the sensitivity and the negative predictive value are important. Indeed, as enforcement is including a positive GMO identification step, false positives due to high sensitivity will/can be corroborated in the second round of analyses where event-specific GMO detection methods are applied. The negative predictive value is crucial in assuring that no positives are being missed in the screening.

It can thus be concluded that these SYBR®Green qPCR methods, when considered separately, perform appropriately for screening of GM events at levels as low as 0.1 % or estimated target copies of GM material present in a sample.

Evaluation of the Performance of the CoSYPS Decision Support System

The CoSYPS DSS provided to each participant laboratory generates the list of possible GM events present in the analyzed sample based on the combined outcome of the qPCR analyses. In Table 5, the DDS outcomes of the repetitions for each marker are listed. Upon correcting the analytical results as described above, the correct list of possible GM events present in the GM unknowns was obtained in 100 % of the analyses. Note that only in one case (T_{nos} for unknown U4), the combined analysis resulted in indicating the marker to be present “at LOD”, instead of “below LOD”. In such cases, follow-up analyses will be required to determine the origin of these minor signals.

Consequently, the outcomes of the DSS analysis of the CoSYPS screening results matched very well the expected results. Indeed, due to its strictly mathematical interpretation,

Table 3 Evaluation of all PCR analyses of the GM unknowns of the inter-laboratory trial (after re-reading)

Sample	Test outcome	Plant	ADH alt	STLM (lectin)	CRU	P35S short	Tnos	CP4 epsps	CryIAb	PAT	BAR
Unknown 4 (0.1 % GTS-40-3 soy)	Expected outcome	+	–	+	–	+	+	+	–	–	–
	Pos	72	0	66 ^a	1	72	70	1	0	0	0
	Neg	0	72	0	71	0	2	71	72	72	72
	Spec/sens	100 %	100 %	100 %	99 %	100 %	99 %	99 %	100 %	100 %	100 %
Unknown 5 (2 % Bt11 maize)	Expected outcome	+	+	–	–	+	+	–	+	+	–
	Pos	71	71	4	1	72	71	4	70	71	0
	Neg	1	1	68	71	0	1	68	2	1	72
	Spec/sens	99 %	99 %	94 %	99 %	100 %	99 %	94 %	97 %	99 %	100 %
Unknown 6 (0.1 % Bt11)	Expected outcome	+	+	–	–	+	+	–	+	+	–
	Pos	72	72	1	0	72	71	2	71	69	1
	Neg	0	0	71	72	0	1	70	1	3	71
	Spec/sens	100 %	100 %	99 %	100 %	100 %	99 %	97 %	99 %	96 %	99 %
Unknown 7 (0.1 % GT73 rape)	Expected outcome	+	–	–	+	–	–	+	–	–	–
	Pos	72	0	4	72	0	4	72	4	0	2
	Neg	0	72	68	0	72	68	0	68	72	70
	Spec/sens	100 %	100 %	94 %	100 %	100 %	94 %	100 %	94 %	100 %	97 %
Unknown 8 (0.9 % RF3 rape)	Expected outcome	+	–	–	+	–	+	–	–	–	+
	Pos	65	1	4	66	1	66	1	2	1	66
	Neg	1	65	62 ^a	0	65	0	65	64	65	0
	Spec/sens	98 %	98 %	94 %	100 %	98 %	100 %	98 %	97 %	98 %	100 %
Unknown 9 (2 % RF3 rape, 1 % GTS-40-3 soy)	Expected outcome	+	+	+	+	+	+	+	–	–	+
	Pos	72	72	72	0	72	72	1	71	72	0
	Neg	0	0	0	72	0	0	71	1	0	72
	Spec/sens	100 %	100 %	100 %	100 %	100 %	100 %	99 %	99 %	100 %	100 %
Unknown 10 (0.1 % GT73 rape, 0.5 % MON810 maize)	Expected outcome	+	+	–	+	+	–	+	+	–	–
	Pos	72	72	5	72	72	1	71	69	1	3
	Neg	0	0	61 ^a	0	0	71	1	3	71	69
	Spec/sens	100 %	100 %	92 %	100 %	100 %	99 %	99 %	96 %	99 %	96 %

For each unknown, the GMO is indicated between brackets and the expected outcome is indicated as “+” when the marker is present, as “–” when absent. A sensitivity measure was calculated when a marker is expected to be present; a specificity measure when the marker is expected absent. Abbreviations for the markers are as described in Table 1

Applied formulae: sensitivity (%)=(Pos/(Pos + Neg)) × 100; specificity (%)=(1–(Neg/(Pos + Neg))) × 100

^a Missing data due to failure of the analysis or lack of materials

based on prime-number factorization, this fully compliant screening analysis will give the predicted outcome at the GMO level in the CoSYPS decision support (Van den Bulcke et al. 2010).

Discussion

The results in the inter-laboratory trial demonstrate that the SYBR®Green qPCR methods used in CoSYPS system represent very sensitive GMO detection methods. All SYBR®Green qPCR methods used in the trial indeed detect their respective targets correctly (positive predicting values >97 %) while giving only very few to no false negative results

(negative predicting values >95 %). When combined with a DSS, these SYBR®Green qPCR methods represent a versatile, highly performant and reliable GMO detection approach.

Although all participants scored the CoSYPS system as a simple, easy-to-use system and the provided protocols as very clear, a considerable number (about 5 %) of the analytical runs were not analyzed exactly as described in the protocol. Re-reading of the runs using the same software applied by the laboratories showed that the main deviations were 1) not setting the threshold according to the instructions in the provided protocol and 2) not setting the threshold for every method separately. While the first deviation mainly leads to too high C_t values, the second one may also result in erroneous data. However, both deviating practices resulted in false

Table 4 Sensitivity, specificity, positive and negative predictive value of the PCR methods applied in CoSYPS based on the analysis of the GM unknowns of the inter-laboratory trial (after re-reading)

Expected outcome	Test outcome	Formula	ADH alt	STLM (lectin)	CRU	P35S short	Tnos	CP4 epsps	CryIAb	PAT	BAR	Overall
Pos	Pos	<i>a</i>	287	138	210	352	350	214	281	212	66	2,110
Pos	Neg	<i>b</i>	1	0	0	8	4	2	5	3	0	23
Neg	Pos	<i>c</i>	1	16	1	1	5	8	6	3	5	46
Neg	Neg	<i>d</i>	209	332	287	137	139	274	204	351	355	2,288
Sensitivity (%)		$(a/(a + c)) \times 100$	99.7 %	100.0 %	100.0 %	97.8 %	98.9 %	99.1 %	98.3 %	98.6 %	100.0 %	98.9 %
Specificity (%)		$(1 - (b/(b + d))) \times 100$	99.5 %	95.4 %	99.7 %	99.3 %	96.5 %	97.2 %	97.1 %	99.2 %	98.6 %	98.0 %
Positive Predictive Value (%)		$(a/(a + b)) \times 100$	99.7 %	100.0 %	100.0 %	97.8 %	98.9 %	99.1 %	98.3 %	98.6 %	100.0 %	98.9 %
Negative Predictive value (%)		$(1 - (c/(c + d))) \times 100$	99.5 %	95.4 %	99.7 %	99.3 %	96.5 %	97.2 %	97.1 %	99.2 %	98.6 %	98.0 %

Abbreviations for the markers are as described in Table 1

a denotes the number of analyses that are expected to contain the marker and scored positively, *b* is the number of analyses that are expected to contain the marker but scored negatively, *c* is the number of analysis that do not contain the marker but scored positively, and *d* number of analysis that do not contain the marker and scored negatively

positive and false negative outcomes. In a very few cases, no results were obtained due to a technical failure (not further specified).

After re-reading, the large majority of the false positives and negatives could be resolved. This indicates that CoSYPS, though considered an easy system, merits experimental and theoretical training before being applied in GMO analysis. Indeed, in a separate small-scale trial with only five laboratories who had received a theoretical and practical CoSYPS training previously, the obtained results were close to perfect (data not shown).

Overall, the SYBR®Green qPCR methods allowed to correctly detect three major crop markers for soy, maize and rapeseed, six main GMO markers (P35S, Tnos, CP4 epsps, CryIAb, PAT and BAR) and a generic plant marker (RbCL).

Both in the large- and the small-scale trials, the targets of some GMOs present at 0.1 % levels were reliably detected. Recalculated in absolute estimated copy numbers, in these trials the SYBR®Green qPCR methods detected as little as eight (CP4 epsps), 17 (BAR), 23 (P35S, Tnos and CryIAb) and 95 (PAT) copies in a total of 25 ng DNA extract. In separate reports, all methods were previously shown to

Table 5 Overview of the analysis of the outcome obtained by the combined CoSYPS decision support per marker (*n*=6) on all samples in the inter-laboratory trial

PER method												
		Plant	ADH alt	SLTM (lectin)	CRU	P35S short	Tnos	CP4 epsps	CryIAb	PAT	BAR	
	Total	83	83	83	83	83	83	83	83	83	83	
	Correct	83	83	83	83	83	82	83	83	83	83	
	wrong	0	0	0	0	0	1	0	0	0	0	
	%	100 %	100 %	100 %	100 %	100 %	99 %	100 %	100 %	100 %	100 %	
PER sample												
	# Runs	Plant	ADH alt	SLTM (lectin)	CRU	P35S short	Tnos	CP4 epsps	CryIAb	PAT	BAR	% correct
Unknown 4	12	12	12	12	12	12	11	12	12	12	12	99 %
Unknown 5	12	12	12	12	12	12	12	12	12	12	12	100 %
Unknown 6	12	12	12	12	12	12	12	12	12	12	12	100 %
Unknown 7	12	12	12	12	12	12	12	12	12	12	12	100 %
Unknown 8	11	11	11	11	11	11	11	11	11	11	11	100 %
Unknown 9	12	12	12	12	12	12	12	12	12	12	12	100 %
Unknown 10	12	12	12	12	12	12	12	12	12	12	12	100 %

“Correct” means that the markers included in the unknown were indicated correctly as “above LOD” when present, and “at or below LOD” when absent, applying the decision criteria set in Materials and Methods. Abbreviations for the markers are as described in Table 1

efficiently detect less than 20 copies of their respective targets (Barbau-Piednoir et al. 2010; Barbau-Piednoir et al. 2012; Mbongolo Mbella et al. 2011). Here, in these inter-laboratory trials, several GMOs present at a 0.1 % mass level in the GM unknowns (e.g., MON810 maize and GT73 rapeseed) were efficiently detected. Thus, CoSYPS is highly suitable to screen for the presence of GMOs complying with the recent so-called “Low level Presence” EU regulation EU/619/2011 (European 2011).

Recently a number of reports have documented the inter-laboratory testing of a several probe-based multitarget qPCR systems, both simplex qPCR methods and multiplex qPCR methods (e.g., see Reiting et al. 2007; Waiblinger et al. 2008; Grohmann et al. 2009). These methods allow detecting GMOs as efficiently in test samples as the SYBR®Green qPCR methods used in CoSYPS. Due to the inherent need of a fluorescently labeled probe as the means of detection, the combination of three nucleotides in one test does in most cases not allow detecting multiple related GM targets within a single test. Although such a one-to-one correlation increases specific recognition at the DNA sequence level, it hampers broad-range screening approaches wherein related sequences can be detected by the same test (see, e.g., the SYBR®Green qPCR methods for CryIAb and CP4 epsps used in this study). In the latter, the number of assays to be included in a screening can be reduced. In the advent of new events comprising recombinant sequences with little to no homology with previously registered GMOs, the success of an “open” screening strategy may well be the preferred approach in GMO analysis. The GM marker set used in the CoSYPS setup applied in this 2009 inter-laboratory trial covers merely 42/48 (=88 %) of the currently EU authorized GMOs (according to the information in the EU Register of authorised GMOs at 27/02/2014; http://ec.europa.eu/food/dyna/gm_register/index_en.cfm). To cover the whole spectrum of GMOs falling under the current EU legislation for commercial applications, 18 screening methods would be required and more are expected to be needed in the future (Broeders et al. 2013).

Considering the variety in sequence nature of the targets of all SYBR®Green qPCR methods presented in this study (generic plant targets, plant species, recombinant sequences), the CoSYPS method development criteria and performance assessment (Barbau-Piednoir et al. 2010; Barbau-Piednoir et al. 2012; Van den Bulcke et al. 2010; Mbongolo Mbella et al. 2011) provides without any doubt a valuable framework for further extending the set of GMO screening qPCR methods operating under the same experimental conditions (see, e.g., Block et al. 2013). It is likely though that in the future larger PCR formats may have to be introduced, simplex PCR methods will have to be combined by multiplexing, or GMO screening applying other technologies such as digital PCR or Luminex may need to be established taking also cost into consideration (Randhawa et al. 2014; Shao et al. 2014;

Brod et al. 2014; Huber et al. 2013; Cottenet et al. 2013). Finally, genome-wide-sequencing/exome-sequencing approaches may ultimately become feasible when cost and reliability of this technology are at the level affordable and required by enforcement measures (Wahler D 2999). For the coming years though, real-time PCR matrix-based screening will still play a central role in GMO enforcement testing. The methods and perhaps even more the molecular targets applied in CoSYPS could represent a starting point in the development as well as the application of GMO screening approaches with these new technologies.

Acknowledgments We thank all members of the laboratories that participated at either of the two inter-laboratory trials in this study: Voedsel en Waren Autoriteit (Nederland), Biogevest-France, Finnish Customs Laboratory (Finland), Institut für Hygiene und Umwelt (Germany), Centro Nacional de Alimentación (Spain), Danish Plant Directorate (Denmark), Istituto Superiore di Sanità (Italy), German National Reference Laboratory for GMO (Germany), Federal Office of Public Health (Switzerland), Tallinn University of Technology (Estonia), Institute for Diagnosis and Animal Health (Romania), National Center of Public Health Protection (Bulgaria) and Instituto Nacional de Engenharia, Tecnologia e Inovação (Portugal). We also thank our colleagues at the Institute of Public Health (Brussels, Belgium) and at the Walloon Agricultural Research Centre (Gembloux, Belgium) for the use of some of their equipment in the testing of the feasibility of the CoSYPS on different PCR devices. We thank Els Vandermassen and Dirk Van Geel for their valuable help in preparing and testing some of the reference materials.

Conflict of Interest Elodie Barbau-Piednoir has no conflict of interest. Pieter Stragier has no conflict of interest. Nancy Roosens has no conflict of interest. Marco Mazzara has no conflict of interest. Cristian Savini has no conflict of interest. Guy Van den Eede has no conflict of interest. Marc Van den Bulcke has no conflict of interest. This article does not contain any studies with human or animal subjects.

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